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Cyclin D3 Sensitizes Tumor Cells to Tumor Necrosis Factor-Induced, c-Myc-Dependent Apoptosis

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c-Myc is an important mediator of apoptosis in cytokine- or serum-deprived cells and sensitizes various cell types to tumor necrosis factor alpha (TNF) cytotoxicity. However, downstream mediators of c-Myc-dependent apoptosis are largely unknown. In this study, we investigated whether one or more cyclins which, like c-Myc, are important regulators of the cell cycle are involved in TNF-induced apoptosis downstream of c-Myc. Cyclin D3 and c-Myc levels in HeLa and fibrosarcoma cells correlated with sensitivity of these cells to TNF-induced apoptosis, as both proteins were highly expressed in TNF-sensitive HeLa D98 cells and HT-1080 fibrosarcoma cells but not in their TNF-resistant counterparts, HeLa H21 and SS-HT-1080 cells, respectively. All other cyclins tested were equally expressed in all tumor cell lines. Reduction in the expression of c-Myc by dexamethasone or inhibition of the transcriptional activity of c-Myc by introduction of a dominant negative form of c-Myc into TNF-sensitive HeLa D98 cells strongly suppressed the expression of cyclin D3 (but none of the other cyclins) and rendered the cells resistant to TNF-induced apoptosis. Conversely, introduction of the c-*myc* gene into TNF-resistant, c-Myc- and cyclin D3-deficient HeLa H21 cells resulted in enhanced cyclin D3 expression and TNF killing. When cyclin D3 expression in HeLa cells was altered by sense or antisense cyclin D3 cDNA, there was a concomitant alteration in their susceptibility to TNF-induced apoptosis without any change in c-Myc levels. Overall, our results show that cyclin D3 sensitizes tumor cells to TNF-induced apoptosis and indicate that the expression of c-Myc and expression of cyclin D3 in HeLa and in HT-1080 fibrosarcoma cells are closely linked.

Tumor necrosis factor alpha (TNF) potently induces apoptotic or necrotic forms of cell death in some tumor cells (6). Different mechanisms can account for TNF-induced killing, including activation of proteases, phospholipases, and sphingomyelinases, poly(ADP)-ribosylation, synthesis of arachidonate metabolites, changes in intracellular calcium, and generation of destructive free radicals (6, 12, 23).

Apoptosis has been hypothesized to be the result of aberrant cell cycle control, a consequence of conflicting growth signals which cause the inappropriate activation of cell cycle genes (36). In agreement with this hypothesis, it was observed that TNF cytotoxicity is associated with DNA replication and cell division (10). Cells arrested in the G_2/M phase of the cell cycle were more vulnerable to TNF, whereas arrest in G_1 induced by transforming growth factor β (TGF-β) or even TNF itself rendered the cells more TNF resistant (5).

The phosphoprotein c-Myc, in partnership with Max, is a sequence-specific transcription factor that is essential for G_1-to-S phase progression in the cell cycle but is oncogenic when overexpressed (14, 38). c-Myc has also been consistently found to mediate apoptosis following c-Myc overexpression, growth arrest, or withdrawal of certain growth stimuli (13). Furthermore, nuclear c-Myc was found to sensitize two human tumor cell lines to TNF-induced apoptosis (27), and even TNF-resistant fibroblasts could be rendered TNF sensitive by the enforced expression of c-Myc (32). Although these studies did not reveal the mechanism of how c-Myc is involved in TNF-induced apoptosis, we found no evidence for defects in the expression of the putative TNF-protective proteins manganous superoxide dismutase (MnSOD), Bcl2, hsp70, and A20 zinc finger protein (27).

The abilities of c-Myc to sensitize cells to apoptosis and to induce cell cycle progression and oncogenesis require its activation by heterodimerization with Max, as well as the presence of the same N-terminal regions of c-Myc, which contain domains necessary for transcriptional transactivation (3, 4, 7, 29, 54). Cell cycle progression is dependent on the coordinated expression of a variety of cyclins, which form complexes with and activate cyclin-dependent kinases (cdks) (20). The activated kinases phosphorylate a number of key substrates, e.g., the retinoblastoma protein (Rb), thereby driving cells through the cell cycle to mitosis. The D-type cyclins appear to play important roles in G_1-phase entry and exit, are synthesized at different times during the cell cycle, and are present in various combinations in a number of cell types studied (20, 26). Cyclins D1 and D2 can activate cdk4 and cdk6 and are putative proto-oncogene products (26), whereas the function of cyclin D3 in the G_1 phase is less well defined. There is evidence that cyclin A is positively regulated by c-Myc (25, 28), and the unscheduled activation of cyclins A, B, and D1 has been linked to apoptosis by a number of investigators (16, 17, 24, 25, 33, 53), but the possible role of cyclin D3 in apoptosis has not been reported.

It seemed plausible from these considerations that inappropriate transcriptional activation by c-Myc of one or more cyclins during normal growth might also be required to sensitize tumor cells to TNF-induced apoptosis (27). With this in mind, we compared a variety of cyclins in TNF-sensitive and TNF-resistant tumor cells and found no differences in cyclin gene expression, except in the case of cyclin D3 mRNA and protein, which are, like c-Myc, far more abundant in TNF-sensitive cells. We provide several independent lines of evidence that the expression of cyclin D3 in tumor cells is closely linked to

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the expression of c-Myc and that cyclin D3 sensitizes tumor cells to TNF-induced, c-Myc-dependent apoptosis.

MATERIALS AND METHODS

Cell culture and biological reagents. Human HeLa H21 and D98 cells (27) and the human fibrosarcoma cell line HT-1080 (35) were maintained in RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum, 10 mM glutamine, and 50 μg each of streptomycin and penicillin per ml in a humidified incubator containing 5% CO_2. The PAI-2-transfected cell line SS-HT-1080 (35) was cultured under the same conditions except that 0.2 mg of G418 (Life Technologies, Inc.) per ml was added to the culture medium. The TNF used was recombinant human TNF with a specific activity of 4×10^7 U/mg of protein. Water-soluble dexamethasone (Dex) and bromodeoxyuridine (BrdU) were purchased from Sigma Chemical Co. Monoclonal antibodies against cyclins A, B, E, D1, D2, and D3 were purchased from Santa Cruz Biotechnology. The monoclonal antibody for c-Myc (9E10) was purchased from Oncogene Science.

cDNA probes and Northern (RNA) blot analysis. Complementary DNAs encoding cyclins A, B, and E were kindly provided by P. D. Robbins, University of Pittsburgh. cDNAs encoding cyclins D1, D2, and D3 (26) were generated by PCR and confirmed by sequence analysis. The cDNAs for c-*myc* and β-actin were obtained previously (27). Northern blot analysis was carried out as described previously (27).

Expression plasmids, cell transfections, and chloramphenicol acetyltransferase (CAT) assays. The cDNA for the C-terminal half of c-Myc (containing all of exon 3; designated C-t Myc) was generated by PCR. The amplified product was then cloned into the *Eco*RI-*Bam*HI site of the mammalian expression vector pxj41-neo under the control of the cytomegalovirus promoter (57). A full-length c-*myc* cDNA which was described previously (27) was cloned into the expression vector pcDNA3 (Invitrogen Corp.) under the control of the cytomegalovirus promoter. A full-length cyclin D3 cDNA was cloned into the *Bam*HI site of pcDNA3. Sense or antisense orientation was confirmed by restriction enzyme analysis.

HeLa D98 and H21 cells were transfected with 20 μg of plasmid DNA by electroporation using a Bio-Rad gene pulser. After 48 h, cells were trypsinized and reseeded in medium containing 800 μg of G418 (Gibco, BRL) per ml. After 2 to 3 weeks, individual colonies were picked and propagated for further analysis. All stably transfected cell lines were maintained in medium containing 200 μg of G418 per ml. As a control, both cell lines were transfected with the corresponding vector DNA alone.

For CAT assays, cells were transfected in triplicate with 5 μg of either of the two MinCAT reporter plasmids with or without a fourfold repeat of the CAC GTG Myc/Max binding sequence (+M4 or −M4 construct) (34) by electroporation with a Bio-Rad gene pulser. After 40 h, cell extracts were prepared and CAT assays were performed by standard procedures, using 100 μg of total protein per assay (19). Relative transfection efficiencies were determined by parallel transfection of the cells with 5 μg of a Rous sarcoma virus-CAT reporter plasmid.

Preparation of nuclear extracts and cellular lysates. Nuclear extracts were prepared as described previously (27) except that a protease inhibitor cocktail (Boehringer Mannheim) was added to all buffers to prevent proteolytic cleavage of highly unstable proteins. For preparation of cellular lysates, 5×10^6 cells were washed twice with cold phosphate-buffered saline and lysed for 30 min at 4°C in 200 μl of 1% Nonidet P-40 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor cocktail. Nuclear and cell debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. Protein concentrations were determined with the Bio-Rad protein assay.

Western blotting (immunoblotting), immunoprecipitation, and cdk assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed essentially as described previously (27) except that the secondary antibodies were horseradish peroxidase conjugated (Sigma). Filters were developed with the ECL (enhanced chemiluminescence) detection system (Amersham International plc) and exposed for short times (5 to 30 s) to X-ray film. In contrast to the previously described alkaline phosphatase detection method (27), the more sensitive ECL detection system enabled us to show low levels of c-Myc in HeLa H21 cells. To quantitate proteins on Western blots, autoradiographs were scanned with a Bio Image system (Visage 110). Immunoprecipitation and cdk assay were performed as described previously (39). The cdk substrate glutathione *S*-transferase (GST)–RbΔ1-300 (lacking the N-terminal 300 amino acids) was provided by P. Singh and W. Hong of our institute.

TNF cytotoxicity assay. The TNF cytotoxicity assays were performed as described previously (27).

RESULTS

Elevated cyclin D3 expression in TNF-sensitive tumor cells correlates with increased cdk activity. We and others showed that the nuclear oncoprotein c-Myc sensitizes various tumor and other cell lines to the cytotoxic (apoptotic) activity of TNF (27, 32). We also reported that c-Myc was present in nuclear extracts of TNF-sensitive HeLa D98 cells and HT-1080 fibrosarcoma cells but was undetectable in TNF-resistant HeLa H21 and SS-HT-1080 fibrosarcoma cells (27). To identify possible proteins in the apoptotic pathway of TNF that act downstream of c-Myc, we compared the mRNAs of TNF-sensitive and TNF-resistant tumor cells for the expression of various cyclins which, like c-Myc, are involved in the regulation of the cell cycle. Total RNA from 70 to 80% confluent HeLa D98 and H21 cells was prepared and hybridized to DNA probes for cyclins A, B1, E, D1, D2, and D3 and, as a control, β-actin. The two HeLa cell lines expressed similar amounts of mRNAs for cyclins A, B1, E and D2, but cyclin D2 mRNA could not be detected in either cell line. Only cyclin D3 mRNA was differentially expressed in the two HeLa cell lines (Fig. 1A). In contrast to a high cyclin D3 mRNA expression in TNF-sensitive HeLa D98 cells, cyclin D3 mRNA was poorly expressed in TNF-resistant HeLa H21 cells (Fig. 1A). Cyclin D3 mRNA was also detected in TNF-sensitive HT-1080 cells but not in TNF-resistant SS-HT-1080 cells (data not shown).

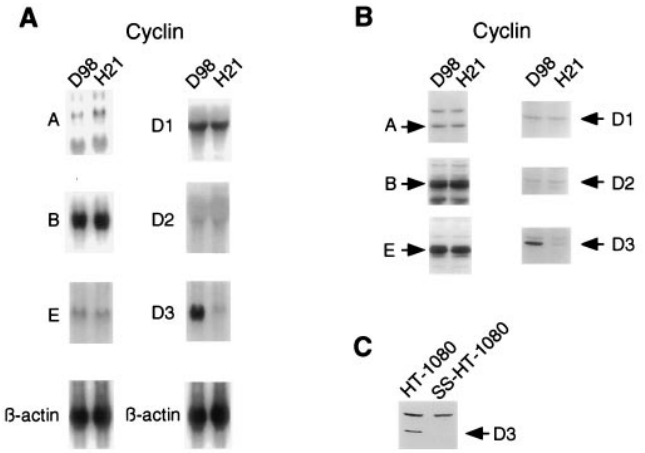

FIG. 1. Elevated cyclin D3 expression in TNF-sensitive HeLa D98 and HT-1080 fibrosarcoma cells. (A) Northern blot analyses of RNAs (10 μg per lane) from HeLa D98 cells and HeLa H21 cells. Total cellular RNA was prepared as described previously (27) and hybridized to the various cyclin cDNA probes (cyclins A, B, E, D1, D2, and D3). After stripping, the filters were rehybridized with a β-actin probe. Autoradiographs were exposed for 3 days. (B) Western blot analyses of various cyclins in total cell extracts (50 μg protein per lane) from HeLa D98 cells and HeLa H21 cells. The arrows indicate the full-length cyclin proteins. (C) Western blot analysis of cyclin D3 in HT-1080 and SS-HT-1080 fibrosarcoma cells.

To determine whether cyclin D3 protein was also differentially expressed in these tumor cells, cell extracts were prepared, and the amounts of the various cyclin proteins were determined by Western blot analysis. The results paralleled those obtained by Northern RNA analysis, demonstrating that cyclin D3 protein was abundant only in extracts of TNF-sensitive HeLa D98 cells (Fig. 1B). In addition, cyclin D3 protein (like c-Myc) was detectable in the TNF-sensitive fibrosarcoma cell line HT-1080 but not in the TNF-resistant fibrosarcoma line SS-HT-1080 (Fig. 1C), indicating a close correlation between c-Myc and cyclin D3 expression in these tumor cells. All other cyclins tested were equally well expressed in both HeLa cell lines (Fig. 1B) as well as in the two fibrosarcoma cell lines (data not shown). Since the cyclin D2 antibody cross-reacts with cyclin D1 protein, it is unclear whether the faint bands observed with this antibody represent cyclin D2 or cyclin D1 protein (Fig. 1B). However, the two cell lines expressed similar amounts of this protein.

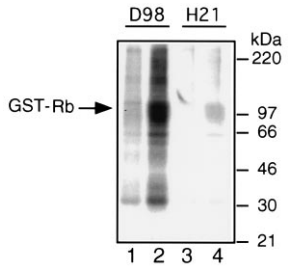

FIG. 2. Elevated cyclin D3 in HeLa D98 cells correlates with increased cdk activity. Total cell extracts from HeLa D98 cells (lanes 1 and 2) and HeLa H21 cells (lanes 3 and 4) were subjected to immunoprecipitation with a cyclin D3 antibody, and the precipitates were tested for kinase activity toward GST (lanes 1 and 3) and GST–RbΔ1-300 (lanes 2 and 4). The reactions were analyzed by SDS-PAGE and exposed to X-ray film.

D-type cyclins are regulators of cdk activity, and recently it was shown that elevated levels of cyclin D1 associated with an increased cdk4 activity induce neuronal cell death (33). To determine whether the increased cyclin D3 levels in HeLa D98 cells were also associated with an increased cdk activity, cyclin D3 was immunoprecipitated from cell lysates of both HeLa cell lines, and the precipitates were tested for kinase activity toward GST-Rb. Since the N-terminal region of Rb is not essential for this kinase reaction, a truncated Rb (RbΔ1-300) lacking the N-terminal 300 amino acids was used for the experiment. As shown in Fig. 2, cdk activity was much greater in cyclin D3 immunoprecipitates of HeLa D98 cells (lane 2) than in those of HeLa H21 cells (lane 4). As a control, GST was not phosphorylated by cyclin D3 precipitates from either HeLa D98 cells (lane 1) or HeLa H21 cells (lane 3). Thus, elevated cyclin D3 levels in HeLa D98 cells are accompanied by an increased cdk activity.

Evidence that the expression of c-Myc and the expression of cyclin D3 are linked. As the high abundance of cyclin D3 protein in HeLa D98 and HT-1080 cells correlated well with c-Myc expression and TNF sensitivity, we examined the effect of altering c-Myc levels on cyclin D3 expression. We demonstrated previously that Dex reduces nuclear c-Myc levels and renders HeLa D98 cells resistant to TNF-induced apoptosis (27). Therefore, HeLa D98 cells were incubated with Dex, and the cell lysates were examined by Western blotting with an anti-cyclin D3 antibody. Cyclin D3 expression in the Dex-treated cells declined in a time-dependent manner: a 12-h treatment with Dex resulted in a 30% reduction in cyclin D3 protein, which was further reduced by over 80% after 36 to 48 h (Fig. 3A, lower panel). A 12-h Dex treatment of HeLa D98 cells resulted in the maximum 70 to 80% reduction in c-Myc protein, clearly indicating that the decline in cyclin D3 follows the reduction in c-Myc (Fig. 3A, upper panel). The same results were obtained when TNF-sensitive HT-1080 fibrosarcoma cells were treated with Dex (data not shown). Consistent with our previous report (27), Dex treatment accelerated growth of HeLa D98 cells, ruling out the possibility that the reduction in c-Myc and cyclin D3 protein levels was caused by growth suppression. The expression of cyclins A, B1, E, D1, and D2 in both HeLa cell lines was unaffected by Dex treatment (Fig. 3B). As Dex treatment was shown previously to reduce nuclear c-Myc levels and to render HeLa cells resistant to TNF cytotoxicity (27), these results not only suggest that the expression of cyclin D3, but none of the other cyclins tested, is linked to the expression of c-Myc but also raise the possibility that cyclin D3 is involved in TNF-induced apoptosis.

Transfection of the 3′ end of c-*myc* renders HeLa D98 cells resistant to TNF killing and decreases cyclin D3 expression. It has previously been shown that the C-terminal region of c-Myc (C-t Myc) is essential for sequence-specific DNA binding and contains the binding site for Max (14). However, to exert its biological functions in cell proliferation and apoptosis, the N-terminal transactivation domain of c-Myc is required. Therefore, we examined the effects of a truncated c-Myc protein stably expressed in TNF-sensitive HeLa D98 cells on TNF susceptibility and cyclin D3 expression. C-t *myc* was cloned into the mammalian expression vector pxj41-neo (57) under the control of the cytomegalovirus promoter and stably transfected into HeLa D98 cells. Three independent D98/C-t Myc clones were chosen, none of which showed any obvious alterations in growth rates compared with the parental HeLa D98 cells. When treated with various TNF concentrations in the absence of cycloheximide (Chx), the D98/C-t Myc clones were found to have become almost completely resistant to the cytotoxic activity of TNF (Fig. 4B). Whereas the highest TNF concentrations, 10 and 100 ng/ml, specifically killed >50 to 60% of parental HeLa D98 cells and D98 cells transfected with vector alone, these same TNF concentrations killed only ~5 to 16% of the three D98/C-t Myc clones (Fig. 4B). A remarkable TNF resistance over a wide range of TNF concentrations was also achieved in all three D98/C-t Myc clones when the TNF cytotoxicity assay was performed in the presence of Chx (Fig. 4A). Essentially the same results were obtained when C-t *myc* was transfected into the TNF-sensitive fibrosarcoma cell line HT-1080 (data not shown).

These results imply that the mechanism by which overexpressed C-t Myc confers resistance to TNF in HeLa D98 cells is through a dominant negative effect on the transcriptional activity of the endogenous c-Myc protein (3, 7). To confirm this possibility, the + or − M4 construct (34) was transfected into HeLa D98 cells, the three D98/C-t Myc clones, and the D98/vector cells, and CAT reporter activity was measured. All three D98/C-t Myc clones showed a two- to fivefold inhibition of CAT activity compared with the parental HeLa D98 cells (Fig.

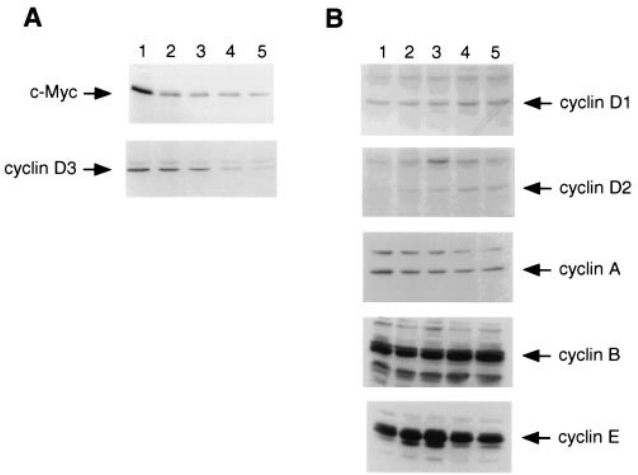

FIG. 3. Reduction in c-Myc protein by Dex leads to a decrease in cyclin D3 protein in HeLa D98 cells. (A) Western blot. Nuclear extracts (upper panel; 10 μg of protein per lane) or total cell extracts (lower panel; 50 μg of protein per lane) from HeLa D98 cells treated with 1 μM Dex for 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 36 h (lane 4), or 48 (lane 5) were subjected to SDS-PAGE and analyzed by immunoblotting for c-Myc (upper panel) or cyclin D3 (lower panel). (B) Western blot. Total cell extracts (50 μg of protein per lane) from HeLa D98 cells treated as for panel A were subjected to SDS-PAGE and analyzed by immunoblotting for expression of the indicated cyclins.

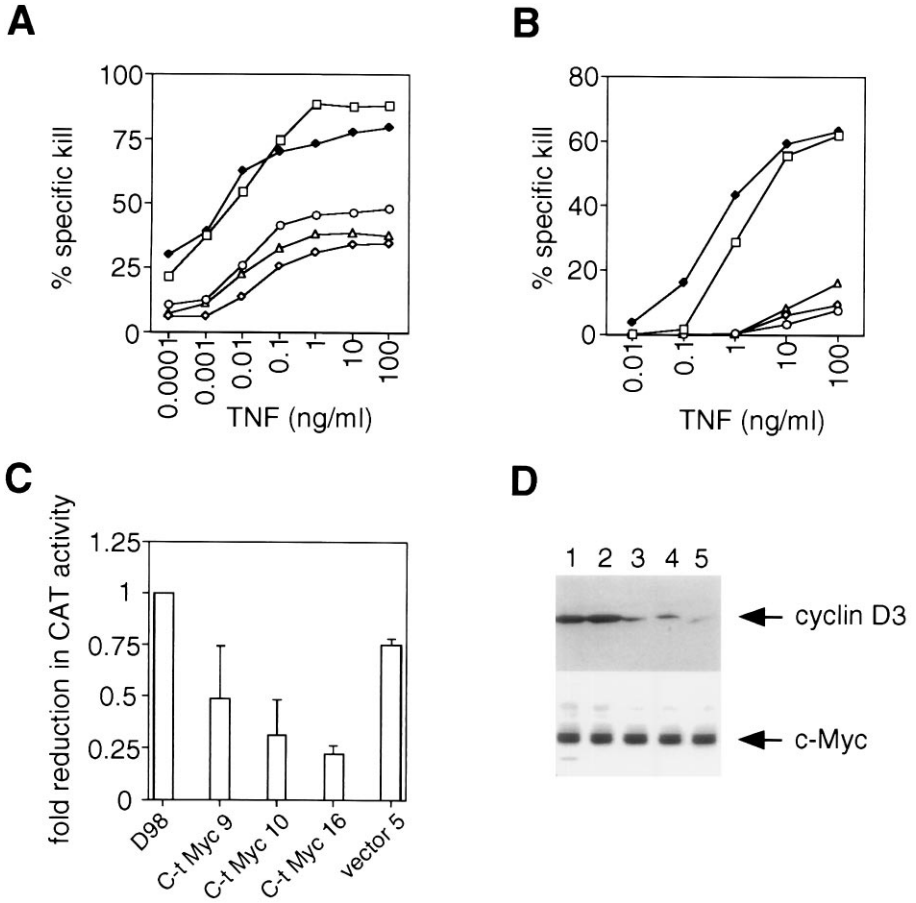

FIG. 4. Suppression of c-Myc transcriptional activity results in TNF resistance and decrease in cyclin D3 protein. (A and B) TNF cytotoxicity assay. HeLa D98 parental cells (squares), D98/vector cells (filled diamonds), D98/C-t Myc 9 cells (circles), D98/C-t Myc 10 cells (triangles), and D98/C-t Myc 16 cells (open diamonds) were incubated at 37°C in the presence (A) and absence (B) of Chx (10 μg/ml) for 16 and 72 h, respectively, in 10-fold dilutions of TNF. Values shown represent the averages of four independent experiments with duplicate determinations. (C) CAT assay (see Materials and Methods). CAT activities are expressed as fold reduction in CAT activity compared with parental HeLa D98 control cells, in which the background reporter activities obtained with the −M4 CAT construct were subtracted for each cell line. Values shown represent the averages of three independent experiments. (D) Western blot. Total cell extracts (upper panel; 50 μg of protein per lane) or nuclear extracts (lower panel; 10 μg of protein per lane) from HeLa D98 cells (lane 1), D98/vector cells (lane 2), D98/C-t Myc 9 cells (lane 3), D98/C-t Myc 10 cells (lane 4), and D98/C-t Myc 16 cells (lane 5) were subjected to SDS-PAGE and analyzed by Western blotting with an anti-cyclin D3 antibody (upper panel) or anti-c-Myc antibody (lower panel).

4C). The D98/vector cells showed only an insignificant decrease in CAT activity. These results are consistent with a recent report suggesting that activation of transcription through the Myc-Max complex and not the repression of basal promoters is critical in TNF-induced apoptosis (32).

To determine whether cyclin D3 protein expression is affected by the inhibition of the transcriptional activity of the endogenous c-Myc protein, Western blot analyses were performed. Overexpression of the C-t Myc in HeLa D98 cells led not only to TNF resistance but also to a substantial reduction in cyclin D3 protein levels in all three D98/C-t Myc clones compared with either HeLa D98 or D98/vector cells (Fig. 4D, upper panel). On the other hand, endogenous c-Myc levels remained unchanged in the three D98/C-t Myc clones (Fig. 4D, lower panel). Only cyclin D3 was reduced in the D98/C-t Myc clones: the levels of cyclins A, B, E, D1, and D2 were the same as found in HeLa D98 and D98/vector cells (data not shown). These results clearly demonstrate that the expression of cyclin D3 is closely linked to the activity of c-Myc because the C-terminal truncated c-Myc protein exerts a dominant negative effect on transcriptional activation of c-Myc-responsive genes by competing with full-length endogenous c-Myc for binding to Max (3, 7). Furthermore, these data raise the intriguing possibility that cyclin D3 acts downstream of c-Myc in sensitizing HeLa and HT-1080 fibrosarcoma cells to TNF-induced apoptosis.

BrdU enhances cyclin D3 expression and TNF sensitivity in D98/C-t Myc cells. The transcription factor YY1 has been implicated in transcriptional activation of the c-*myc* gene (49). In agreement with this observation, we found that extracts of the TNF-resistant HeLa H21 and SS-HT-1080 fibrosarcoma cells, which both had undetectable levels of nuclear c-Myc (27), exhibited a much weaker YY1 binding in bandshift analyses compared with their TNF-sensitive counterparts (unpublished observations). In addition, treatment of primary myoblasts with BrdU increased the YY1 protein content and subsequently led to an increase in c-Myc protein (37). We studied the effects of BrdU in our cell lines to address the issue of whether YY1 is involved in c-Myc-dependent apoptosis and, furthermore, to determine whether BrdU would increase c-Myc and concomitantly cyclin D3 levels in HeLa cells. Treatment of D98/C-t Myc cells for 3 days with 30 μM BrdU partially reverted their acquired TNF resistance and made them more susceptible to TNF cytotoxicity (Fig. 5A). TNF sensitivity

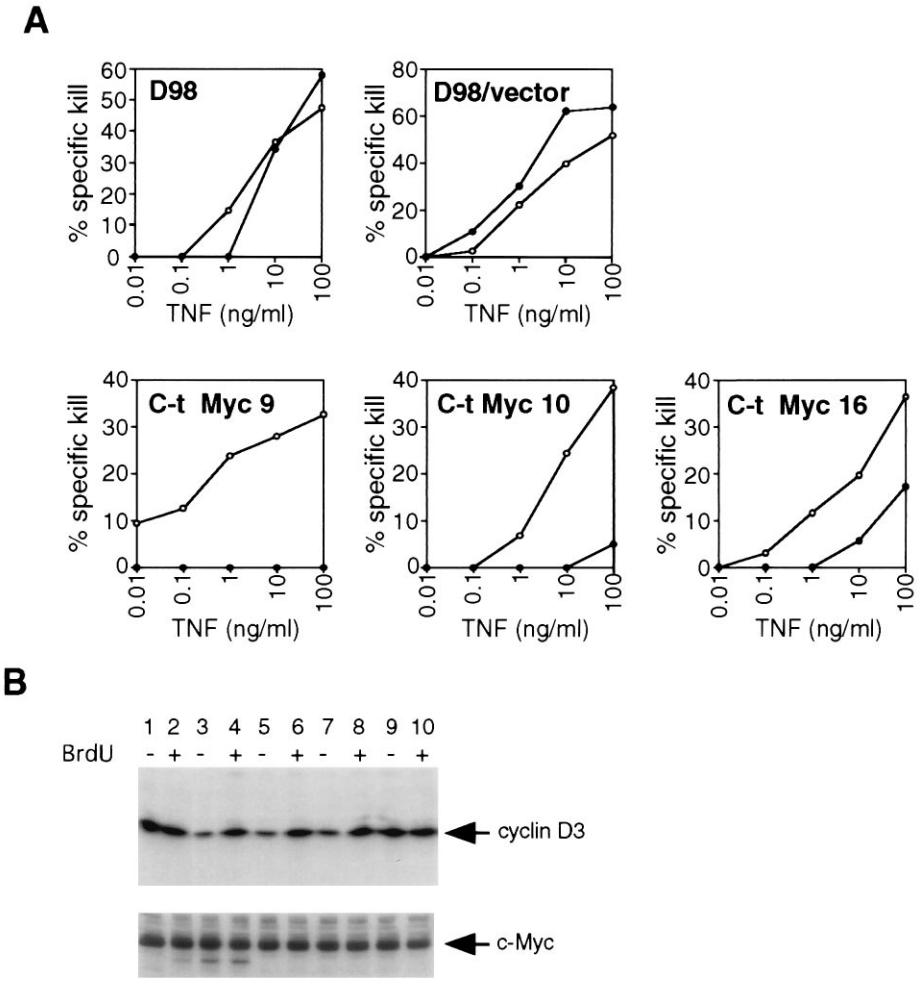

FIG. 5. BrdU treatment reverts acquired TNF resistance of HeLa D98/C-t Myc transfectants and increases cyclin D3 expression. (A) TNF cytotoxicity assay. Control untreated cells (closed circles) and cells treated for 3 days with 30 μM BrdU (open circles) were incubated at 37°C for 72 h in the absence of Chx in 10-fold dilutions of TNF. Values shown represent the averages of three independent experiments with duplicate determinations. (B) Western blot. Total cell extracts (upper panel; 50 μg of protein per lane) or nuclear extracts (lower panel; 10 μg of protein per lane) were prepared from untreated cells (lanes 1, 3, 5, 7, and 9) or BrdU-treated cells (lane 2, 4, 6, 8, and 10). Parental HeLa D98 cells (lanes 1 and 2), D98/C-t Myc 9 cells (lanes 3 and 4), D98/C-t Myc 10 cells (lanes 5 and 6), D98/C-t Myc 16 cells (lanes 7 and 8), and D98/vector cells (lanes 9 and 10) were subjected to SDS-PAGE and analyzed by Western blotting for cyclin D3 (upper panel) or c-Myc (lower panel) expression.

of parental HeLa D98 cells and D98/vector cells remained virtually unchanged after a similar incubation with BrdU (Fig. 5A). Contrary to the earlier findings (37), treatment of HeLa D98 cells, D98/vector cells, and the transfected derivatives with BrdU did not alter the expression of either YY1 protein (data not shown) or endogenous c-Myc protein (Fig. 5B, lower panel), indicating that neither of these proteins is responsible for the reverted TNF sensitivity of BrdU-treated D98/C-t Myc cells. However, the conversion of the TNF-resistant phenotype of D98/C-t Myc cells to the TNF-sensitive phenotype by BrdU treatment was accompanied by a regained cyclin D3 expression in all three D98/C-t Myc clones (Fig. 5B, upper panel). In contrast, cyclin D3 expression in the parental D98 cells or D98/vector cells was not affected by BrdU treatment. Taken together, these results provide further evidence that cyclin D3 is involved in TNF-induced apoptosis in these tumor cells and that the expression of cyclin D3 can be regulated in two distinct pathways: a c-Myc-dependent and a c-Myc-independent pathway.

Transfection of a full-length c-*myc* cDNA renders HeLa H21 cells sensitive to TNF cytotoxicity and results in expression of cyclin D3. To provide further evidence that cyclin D3 expression and c-Myc expression are linked and that cyclin D3 is involved in TNF-induced apoptosis, we stably transfected a full-length c-*myc* cDNA in sense orientation into TNF-resistant HeLa H21 cells, which originally had low levels of both endogenous c-Myc and cyclin D3 protein. Several G418-resistant clones were isolated, and three were analyzed in detail for c-Myc and cyclin D3 expression as well as for TNF susceptibility. Significantly increased levels of c-Myc in H21/Myc clones 7, 67, and 68 were confirmed by Western blotting (Fig. 6B, upper panel). The introduction of the c-*myc* gene into HeLa H21 cells resulted not only in an increase in sensitivity to TNF killing (Fig. 6A) but also in a marked elevation in cyclin D3 protein expression in all three H21/Myc clones (Fig. 6B, lower panel). In contrast, the expression of cyclins A, B1, D1, D2 and E remained unchanged (data not shown). Transfection of the vector alone altered neither the TNF susceptibility of HeLa

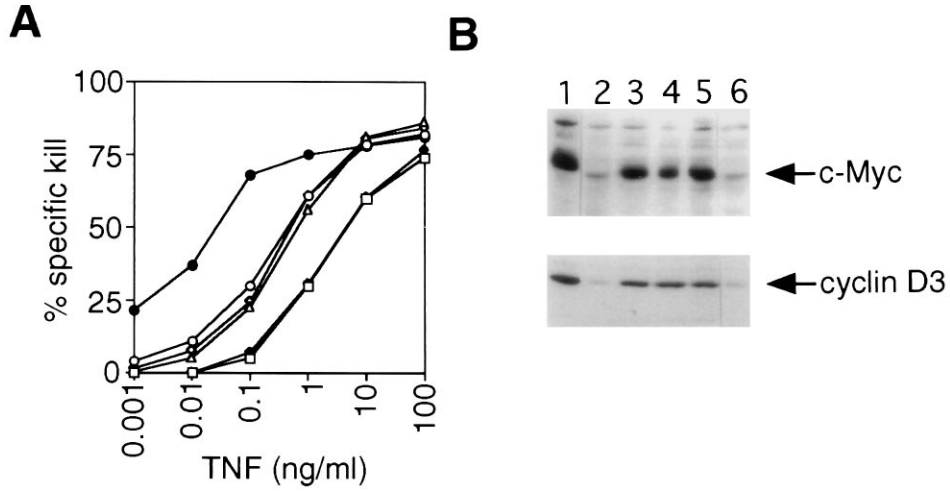

FIG. 6. Transfection of full-length c-*myc* cDNA renders HeLa H21 cells sensitive to TNF cytotoxicity and increases cyclin D3 expression. (A) TNF cytotoxicity assay. HeLa D98 cells (filled circles), HeLa H21 cells (squares), H21/vector cells (filled diamonds), H21/Myc 7 cells (open circles), H21/Myc 67 cells (triangles), and H21/Myc 68 cells (open diamonds) were incubated at 37°C for 16 h in the presence of 10 μg of Chx per ml in 10-fold dilutions of TNF. Values shown represent the averages of six independent experiments with duplicate determinations. (B) Western blot. Total cell extracts (lower panel; 50 μg of protein per lane) or nuclear extracts (upper panel; 10 μg of protein per lane) from HeLa D98 cells (lane 1), HeLa H21 cells (lane 2), H21/Myc 7 cells (lane 3), H21/Myc 67 cells (lane 4), H21/Myc 68 cells (lane 5), and H21/vector cells (lane 6) were subjected to SDS-PAGE and analyzed by immunoblotting with an anti-cyclin D3 antibody (lower panel) or with an anti-c-Myc antibody (upper panel).

H21 cells (Fig. 6A) nor the expression level of cyclin D3 (Fig. 6B, lower panel). Furthermore, none of the H21/Myc transfectants showed any obvious alterations in their growth rates compared with the parental HeLa H21 cell line, suggesting that the increased TNF sensitivity and elevated cyclin D3 expression were not caused by increased growth rates. Taken together, these data provide additional evidence that cyclin D3 sensitizes HeLa cells to apoptosis and that the expression of c-Myc and the expression of D3 are closely linked in these cells.

Although the levels of c-Myc and cyclin D3 in the H21/Myc clones were greatly elevated, they were still lower than in HeLa

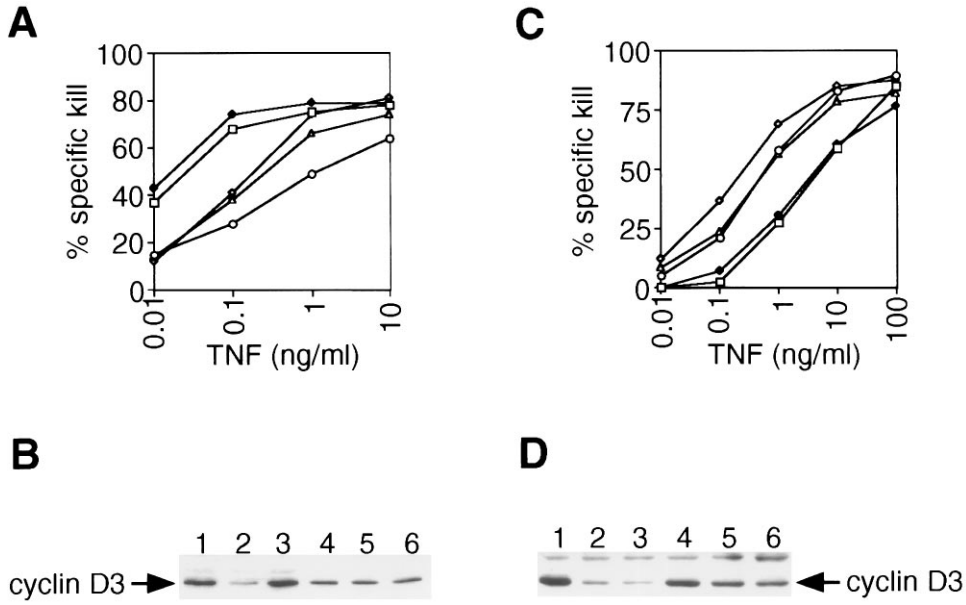

FIG. 7. Cyclin D3 sensitizes HeLa cells to TNF-induced apoptosis. (A) TNF cytotoxicity assay. HeLa D98 cells (squares), D98/vector cells (filled diamonds), D98/A-D3-10 cells (circles), D98/A-D3-38 cells (triangles), and D98/A-D3-40 cells (open diamonds) were incubated at 37°C in the presence of Chx (10 μg/ml) for 16 h in 10-fold dilutions of TNF. Values represent the averages of at least four independent experiments with duplicate determinations. (B) Western blot. Total cell extracts (50 μg of protein per lane) from HeLa D98 cells (lane 1), HeLa H21 cells (lane 2), D98/vector cells (lane 3), D98/A-D3-10 cells (lane 4), D98/A-D3-38 cells (lane 5), and D98/A-D3-40 cells (lane 6) were subjected to SDS-PAGE and analyzed by immunoblotting for cyclin D3 expression. (C) TNF cytotoxicity assay. HeLa H21 cells (squares), H21/vector cells (filled diamonds), H21/D3-31 cells (circles), H21/D3-34 cells (triangles), and H21/D3-42 cells (open diamonds) were incubated at 37°C in the presence of Chx (10 μg/ml) for 16 h in 10-fold dilutions of TNF. Values represent the averages of at least four independent experiments with duplicate determinations. (D) Western blot. Total cell extracts (50 μg of protein per lane) from HeLa D98 cells (lane 1), HeLa H21 cells (lane 2), H21/vector cells (lane 3), H21/D3-31 cells (lane 4), H21/D3-34 cells (lane 5), and H21/D3-42 cells (lane 6) were subjected to SDS-PAGE and analyzed by immunoblotting for cyclin D3 expression.

D98 cells. This finding correlates with the intermediate TNF sensitivity of H21/Myc clones compared with parental HeLa H21 and D98 cells (Fig. 6A).

Specific alterations in cyclin D3 expression change TNF sensitivity of HeLa cells. HeLa D98 cells were transfected with an antisense cyclin D3 cDNA to determine whether a specific downregulation of cyclin D3 would result in an inhibition of TNF killing. Three independent G418-resistant clones (D98/A-D3-10, -38, and -40), which all grew at rates similar to that of the parental HeLa D98 cells, were obtained. The expression of antisense cyclin D3 cDNA in these clones resulted in a 50 to 70% reduction in the synthesis of cyclin D3 protein (Fig. 7B). The reduction in cyclin D3 was accompanied by an up to 50- to 100-fold decrease in sensitivity of the transfected cells to TNF killing in the standard 16-h assay. In this respect, clone D98/A-D3-10 showed the highest TNF resistance even at TNF concentrations of up to 10 ng/ml, whereas clones 38 and 40 were remarkably TNF resistant only at low TNF concentrations (0.01 to 0.1 ng/ml) (Fig. 7A). In contrast, transfection of the HeLa D98 cells with the expression vector alone affected neither cyclin D3 expression (Fig. 7B, lane 3) nor TNF sensitivity (Fig. 7A), demonstrating the specificity of the antisense cyclin D3 cDNA.

It is known that in the presence of Chx, TNF killing is achieved much more rapidly at high TNF concentrations (10 to 100 ng/ml). Since the 50- to 100-fold decrease in TNF sensitivity of two transfectants, D98/A-D3-38 and -40, could be observed only at low TNF concentrations (0.01 to 0.1 ng/ml) in a standard 16-h assay (Fig. 7A), we investigated the possibility that the TNF resistance of these two clones would be more pronounced, even at high TNF concentrations, in a shorter (4-h) TNF assay. In the presence of 100 ng of TNF per ml, which killed 70 to 80% of the parental HeLa D98 cells and D98/vector cells within the first 4 h, only 17 to 45% killing of the D98/A-D3 transfectants was observed at the same point. The 50- to 100-fold decrease in TNF sensitivity of D98/A-D3 clones with low TNF concentrations (0.01 to 0.1 ng/ml) was also consistently achieved when TNF cytotoxicity was assessed after 4 h (data not shown). These experiments clearly demonstrate that significantly reduced cyclin D3 levels in D98/A-D3 transfectants rendered these cells more resistant to the cytotoxic action of TNF, which could be overcome only by using much higher TNF concentrations for a longer period of time. In this respect, the TNF responses of D98/A-D3 and HeLa H21 cells are very similar. In a 4-h TNF assay, HeLa H21 cells remained relatively TNF resistant even at 100 ng of TNF per ml (22% kill), but in a 16-h assay, they became as TNF sensitive as HeLa D98 cells but only at this high TNF concentration (Fig. 6A and 7C).

To confirm the participation of cyclin D3 in apoptosis, we stably transfected a full-length sense cyclin D3 cDNA into HeLa H21 cells. The enhanced expression of cyclin D3 in three independent clones, H21/S-D3-31, -34, and -42, was confirmed by Western blotting (Fig. 7D). None of the H21/S-D3 transfectants exhibited any morphological changes or increased growth rates as was reported for cells overexpressing cyclin D1 (46). However, in agreement with this report, we obtained only transfectants that expressed cyclin D3 at levels intermediate between those in HeLa D98 and H21 cells. Nevertheless, the enhanced expression of cyclin D3 rendered HeLa H21 cells 10- to 20-fold more sensitive to TNF in a 16-h assay (Fig. 7C), which could also be observed at low to moderate TNF concentrations (0.01 to 1 ng/ml) when the TNF cytotoxicity assay was performed for only 4 h (data not shown).

Transfections with sense or antisense cyclin D3 cDNA, which altered TNF sensitivity and cyclin D3 levels in HeLa cells, had no effect on the expression of c-Myc protein (data not shown). These results provide further evidence that cyclin D3 plays an important role in TNF-induced apoptosis downstream of c-Myc.

DISCUSSION

Cyclin D3 sensitizes two different tumor cell types to TNF-induced apoptosis. Several independent lines of evidence presented here show that besides c-Myc, cyclin D3 plays an important role in TNF-induced apoptosis in two different tumor cell types. First, expression of cyclin D3 and c-Myc correlated with TNF sensitivity, as observed in two different cell line pairs (HeLa and fibrosarcoma cells). Second, treatment of HeLa D98 and HT-1080 fibrosarcoma cells with Dex, which confers cellular TNF resistance through downregulation of nuclear c-Myc (27), also resulted in a decrease in the level of cyclin D3 protein. Third, transfection of C-t Myc into HeLa D98 cells repressed the transcriptional activity of endogenous c-Myc by a dominant negative effect, led to a decrease in cyclin D3 protein, and conferred resistance to TNF cytotoxicity. Fourth, enforced expression of c-Myc in HeLa H21 cells, which originally had very low levels of both endogenous c-Myc and cyclin D3 protein, led to an increased expression of cyclin D3 in these cells and rendered them more sensitive to TNF. Finally, HeLa H21 cells overexpressing a cloned cyclin D3 gene became more TNF sensitive, whereas introduction of an antisense cyclin D3 cDNA into HeLa D98 cells resulted in inhibition of TNF cytotoxicity.

c-Myc and D-type cyclins are important factors in the regulation of cell cycle progression (20, 26), and TNF responsiveness of cells has been linked to particular cell cycle phases (5, 10). However, despite their significant differences in cyclin D3 and c-Myc expression, all parental HeLa and HT-1080 fibrosarcoma cell lines grew at comparable rates. Furthermore, none of the various transfectants showed morphological changes or any abnormal growth behavior compared with their parental cell lines. These observations not only suggest that minimal threshold levels of c-Myc and cyclin D3 are sufficient to drive these cells through the cell cycle but also, and more importantly, imply that the observed changes in TNF responsiveness of the transfected cell clones are not caused by altered growth patterns.

There have been several reports indicating that c-Myc regulates expression of cyclins A, D1, and E (9, 25, 28, 43), but only the regulation of cyclin A by c-Myc has been confirmed (25, 28); the involvement of c-Myc in the regulation of cyclins D1 and E is still unclear (9, 25, 28, 43). Elevated cyclin A levels have been shown to play an important role in c-Myc-induced apoptosis in Rat1a fibroblasts; however, no information about the expression of cyclin D3 was provided (25). Therefore, it would be very interesting to determine whether cyclin D3 is also involved in sensitizing Rat1a fibroblasts harboring a hormone-inducible c-Myc–estrogen receptor chimera to TNF-induced apoptosis (32). Furthermore, cyclin D1 has been implicated as an essential mediator of neuronal cell death (17, 33), and deregulated expression of cyclin B or the premature activation of its associated kinase, cdc2, can trigger activation-induced T-cell death and a serine protease-induced apoptotic process, respectively (16, 52). In our system, however, it seems unlikely that cyclins A, D1, and E are regulated by c-Myc and that cyclins A, B, and D1 participate in TNF-induced apoptosis because these proteins were equally expressed in TNF-sensitive and TNF-resistant cell lines, and the various treatments that affected cyclin D3 expression and changed TNF susceptibility of these cells had no influence on cyclin A, B, D1, and E

expression. Thus, of all of the cyclins tested, only cyclin D3 appears to be involved in TNF-induced apoptosis in the cell lines examined.

The role of cyclin D3 in apoptosis. What role does cyclin D3 play in the response to TNF, and how does it mediate apoptosis? The D-type cyclins specifically complex with and activate the kinases cdk4 and cdk6, and cyclins D2 and D3 may in addition complex with cdk2 (15, 31). The D-type cyclins complexed with these cdks are, together with c-Myc, among the major regulators of events in the G_1 phase (20, 26). Cells can proceed into S phase only after phosphorylation of Rb, predominantly by D-type cyclins and their associated kinase cdk4 (55). In addition, cyclin D3 can also bind directly to the hypophosphorylated form of the Rb gene product in the absence of cdks (11, 15, 30), and there was a high level of cyclin D3 associated with Rb-related proteins in differentiated myotubes with little or no kinase activity (31). The ability of Rb to regulate cell growth is tightly linked to its ability to bind to members of the E2F transcription factor family (55).

Inappropriate activation or expression of cyclins A, B, and D1 and of the cdks cdk2 and cdc2 has been linked to apoptosis in a number of studies (16, 17, 25, 40, 52). In addition, there has been recently a demonstration of the direct involvement of cdk4 activation in cyclin D1-induced neuronal apoptosis (33). In agreement with this report, we also found a higher level of cyclin D3-dependent cdk activity in HeLa D98 cells than in HeLa H21 cells, suggesting a possible role for cyclin D3-associated cdk activity in TNF-induced apoptosis. However, further studies involving dominant negative mutants of cyclin D3 which cannot bind cdks but which can still bind Rb (and vice versa) are necessary in order to determine whether cyclin D3 acts through cdks or Rb (or both).

Cyclin D1-induced neuronal cell death was inhibitable by Rb (33), which is consistent with the known antiapoptotic function of Rb (2, 21, 22). There is also evidence that premature release of E2F from Rb causes apoptosis (45, 51, 56). However, any explanation of TNF-induced apoptosis based on these considerations must take into account the well-established fact that TNF kills cells by two different mechanisms: rapidly in the presence of Chx, and slowly in the absence of Chx (6, 48). Thus, in the presence of Chx, cyclin D3 could sensitize cells to TNF-induced apoptosis through inappropriate activation of cdks and/or suppression of the antiapoptotic function of Rb, given the well-known ability of cyclin D3 to independently bind to these proteins (55). Although similar events could occur in the absence of Chx, there is the additional possibility that the inactivation of Rb (by cyclin D3-cdk phosphorylation) in cells treated with TNF alone could lead to the untimely release of the transcription factor E2F, which can induce apoptosis, presumably by participating in the transcription of death genes (2, 21, 22).

In agreement with our results, TGF-β-induced resistance to TNF cytotoxicity (5) was accompanied by downregulation of c-Myc and cyclin D3 expression (8, 18, 42, 47). The TGF-β-induced TNF resistance coincided with a growth arrest of the cells in the G_1 phase of the cell cycle (5), which has been linked to the activity of Rb or other Rb-related proteins (1), because it was shown that both simian virus 40 large T antigen and adenovirus E1A protein overcome TGF-β growth arrest, presumably by forming complexes with Rb and intervening in its growth suppressing function (44). Moreover, the TGF-β-induced G_1 arrest can be overcome by expression of the E2F gene (50).

The expression of c-Myc and the expression of cyclin D3 are closely linked. Does c-Myc regulate cyclin D3 expression, or is c-Myc regulated by cyclin D3? Treatments which altered cyclin D3 levels, such as BrdU or cyclin D3 sense or antisense cDNA transfections, had no effect on c-Myc expression. In contrast, increasing c-Myc levels by transfection resulted in an increase in cyclin D3, whereas decreasing c-Myc transcriptional activity or reducing c-Myc levels with Dex depressed cyclin D3 expression. These data strongly argue against the idea that c-Myc is regulated by cyclin D3 but support the intriguing possibility that c-Myc regulates cyclin D3 expression. However, given the fact that E-box-binding proteins other than c-Myc, e.g., the ubiquitous nuclear protein USF, can bind to the CACGTG sequence (41), one could argue that the transcriptional inhibition achieved with the dominant negative mutant of c-Myc is due to the inhibition of binding of other E-box-binding proteins. Although we cannot completely rule out this possibility, our data showing that cyclin D3 levels increase in HeLa H21 cells transfected with c-*myc* cDNA strongly suggest that the observed effects are due to c-Myc and not to other E-box-binding proteins. However, it is not clear yet whether c-Myc can directly regulate cyclin D3 expression or whether the regulatory activity of c-Myc on cyclin D3 expression is mediated by one or more intervening effectors. The cloning and characterization of the cyclin D3 promoter should yield further insight into this question. If cyclin D3 is regulated by c-Myc, our data suggest that cyclin D3 acts downstream of c-Myc in TNF-induced apoptosis.

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